Chapter – 3

RESULTS
RESULTS

The present study involves data on three aspects, which are depicted below:

(A) Oxidative Stress Study in Serum and Monocyte Cultures

(B) Immunological study by ELISA

(C) A bone markers and osteoclast study by TRAP, ELISA and Quantitative ‘Real time RT-PCR

In the present study, allicin- a natural antioxidant from garlic, curcumin and resveratrol were employed as natural antioxidants in order to investigate their regulatory effects on various parameters as discussed in the results depicted below. Also, a few experiments were carried out by employing EGCG- a green tea polyphenol (GTP) in the present study.

Prior to any study, since most of the experiments involved in the present study involve PBMC/ monocyte cultures receiving various natural antioxidants like allicin, resveratrol and curcumin, thus an attempt was first made to see any adverse effect, if any, of these compounds. Therefore, MTT cell viability assay and assessment of human housekeeping gene R-18 by ‘real time’ RT-PCR was carried out. Assessment of human housekeeping gene R-18 has been reported in the study elsewhere under bone marker study by ‘real time’ RT-PCR. Also, as evident from Table 8, allicin did not show any adverse effect till 500 ng/ml, while resveratrol and curcumin did not show any adverse effect till 25 µg/ml. Hence, the above said cuts off doses were selected in the present study.
Table 8: Dose response effect of Allicin, Resveratrol and Curcumin on the Viability of Monocytes from Osteoporosis patients

<table>
<thead>
<tr>
<th>Allicin, ng/ml</th>
<th>Viable Cells (% of Control)</th>
<th>Resveratrol, µg/ml</th>
<th>Viable Cells (% of Control)</th>
<th>Curcumin, µg/ml</th>
<th>Viable Cells (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>99</td>
<td>0</td>
<td>98</td>
<td>0</td>
<td>96</td>
</tr>
<tr>
<td>50</td>
<td>97</td>
<td>2</td>
<td>99</td>
<td>2</td>
<td>97</td>
</tr>
<tr>
<td>100</td>
<td>98</td>
<td>5</td>
<td>97</td>
<td>5</td>
<td>98</td>
</tr>
<tr>
<td>250</td>
<td>99</td>
<td>10</td>
<td>98</td>
<td>10</td>
<td>95</td>
</tr>
<tr>
<td>500</td>
<td>98</td>
<td>15</td>
<td>97</td>
<td>15</td>
<td>98</td>
</tr>
<tr>
<td>750</td>
<td>71</td>
<td>20</td>
<td>98</td>
<td>20</td>
<td>99</td>
</tr>
<tr>
<td>1000</td>
<td>68</td>
<td>25</td>
<td>98</td>
<td>25</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>65</td>
<td>30</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>60</td>
<td>40</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>58</td>
<td>50</td>
<td>60</td>
</tr>
</tbody>
</table>

*(n=10; p < 0.001)

Section [A]: A Study Related to Oxidative Stress in Serum and Monocyte Cultures:

1. *Glutathione peroxidase (GPx) activity in serum of osteoporosis patients.*

High oxidative stress is well known to be associated with osteoporosis. Therefore, the antioxidant state of osteoporosis patients was assessed by determining the GPx activity in their sera. Upon comparison to healthy subjects, the GPx activity in serum of patients with osteoporosis was appreciably reduced by around 55.84% as evident from the results where GPx activity in sera of osteoporosis patients (n=30) and healthy controls (n=30) was recorded as 35.34 U/ mg protein and 80.02 U/ mg protein respectively (Fig. 1). All values represent mean ± SE; p < 0.001.
2. **Glutathione peroxidase (GPx) activity in culture supernatants of monocytes.**

Human monocytes having the ability to adhere onto culture plates were obtained from PBMCs of healthy controls as well as patients with osteoporosis. In turn, they were cultured for 24 hours as described in methods, and finally, the supernatants were analyzed for GPx activity. Results show that the patient’s samples exhibited an appreciably suppressed GPx activity (19.15 U/ mg protein) when compared to samples of healthy subjects (62.08 U/ mg protein) (Fig. 2). Thus, from the said data, it seems that antioxidant defense system is altered by around 69.15% in patients with osteoporosis. All values are mean ± SE; *p* < 0.001.

3. **Determination of intramonocyte glutathione (GSH) levels.**

An attempt was made to evaluate the intramonocyte glutathione (GSH, a major thiol antioxidant) levels in 24 hours monocyte cultures of osteoporosis patients (n=20) as well as in healthy individuals (n=20). As is evident in Fig. 3, the intramonocyte GSH levels were significantly down-regulated/suppressed in samples of osteoporosis patients (129.02 pg/ml) in comparison to healthy controls (231.25 pg/ml). The percent decrease was computed to be ~ 44%. The data indicates that antioxidant defense gets suppressed/reduced in osteoporosis patients in comparison to non-osteoporosis healthy subjects. All values are mean ± SE; *p* < 0.001.

4. **Dose response effect of Curcumin and Resveratrol on GPx activity in cultured monocytes from healthy controls and osteoporosis patients.**

Due to the established beneficial role of curcumin and resveratrol as a natural antioxidant in a wide spectrum of pathological conditions, thus an attempt was made to study their comparative effects on antioxidant state in supernatants of 24 hours cultured monocytes from patients with osteoporosis (n=20) and healthy individuals (n=20) which served as controls.

Healthy monocytes serving as controls were therefore separately co-cultured for 24 hours with varying concentrations of curcumin and resveratrol (0, 2, 5, 10, 15, 20 and 25 µg/ml) respectively. Cultures were harvested and supernatants obtained were subjected to GPx activity determination. Insignificant variation in the activity; 73.37,
75.33, 70.83, 77.38, 73.27, 70.99 and 72.12 U/mg protein was recorded for healthy individuals at above mentioned curcumin concentrations respectively (Fig. 4). Similarly, no change was also observed when resveratrol was co-cultured with healthy monocytes. In this case, the GPx activity was recorded as 70.12, 73.98, 76.32, 74.54, 78.12, 70.37 and 74.12 U/mg proteins with 0, 2, 5, 10, 15, 20 and 25 µg/ml respectively (Fig. 4).

On the contrary, in case of osteoporosis patients (Fig. 5), the GPx activity was found to increase in a dose-dependent manner from 33.09 U/mg protein, through 37.12, 45.18, 56.08, 63.76, 65.12 and 68.87 U/mg protein at 0, 2, 5, 10, 20 and 25 µg/ml curcumin respectively. The above results clearly prove curcumin to be also an effective natural antioxidant capable of elevating the depressed antioxidant state in case of osteoporosis. All values are mean ± SE, \( p < 0.001 \) and \( n=8 \) in each study group.

Similarly, resveratrol which is known to be a natural antioxidant as well as an immuno-regulator, showed a high magnitude effect on GPx activity in monocyte culture supernatants of osteoporosis patients.

Monocytes were co-cultured for 24 hours with varying doses of resveratrol (0, 2, 5, 10, 15, 20 and 25 µg/ml). Supernatants when subjected to GPx activity showed progressive up-regulation in a dose-dependent manner from 32.88 U/mg protein through 42.16, 49.67, 60.56, 67.54, 68.32 and 69.33 U/mg protein (Fig. 6). Therefore, resveratrol, together with curcumin proved to be effective herbal antioxidant in osteoporosis. All values are mean ± SE; \( p < 0.001 \).

**Effect of varying doses of Allicin on intramonocyte levels of glutathione (GSH).**

Next, an attempt was also made to probe for any regulatory effect of varying doses of allicin (0, 50, 100, 250, and 500 ng/ml) on intramonocyte GSH levels in 24 hours monocyte cultures (\( n=20 \) each for healthy and osteoporosis patients). Substantially low level (117.66 pg/ml) of intramonocyte GSH was recorded at 0 ng/ml allicin compared to healthy individuals’ monocyte cultures (310.35 pg/ml). An appreciable and dose-dependent up-regulation of intramonocyte GSH levels through 156.76, 201.32, 283.43, 288.11 pg/ml was recorded for osteoporosis patients at 0, 50, 100,
250, and 500 ng/ml allicin (Fig. 7). IC$_{50}$ was computed out to be ~ 100 µg/ml. On the contrary, insignificant variation was observed in case of healthy subjects; 310.05, 305.11, 310.23, 300.54 and 312.32 pg/ml (Fig. 7). Thus, allicin from garlic raised the depressed thiol antioxidant state in osteoporosis study subjects to an appreciable extent. All values are mean $\pm$ SE, $p < 0.001$.

**Dose response effect of Resveratrol and Curcumin on intramonocyte glutathione (GSH) levels**

Also, effect of varying concentrations of resveratrol and curcumin (0, 2, 5, 10, 15, 20 and 25 µg/ml) on intramonocyte GSH levels in 24 hours monocyte cultures was investigated (n=20 each for healthy and osteoporosis patients). Interestingly, significantly reduced levels of intramonocyte GSH were recorded at 0 ng/ml resveratrol (123.65 pg/ml) in case of osteoporosis patients when compared to healthy subjects (310.11 pg/ml), which thereafter, increased dose-dependently through 162.43, 174.09, 266.45, 270.12, 283.65 and 288.12 pg/ml at 2, 5, 10, 15, 20 and 25 µg/ml resveratrol respectively (Fig. 8).

Similarly, curcumin exhibited a dose-dependent amelioration in intramonocyte GSH levels which was of the order of 116.32, 133.21, 161.68, 212.22, 263.12, 278.19 and 286.33 pg/ml at 0, 2, 5, 10, 15, 20 and 25 µg/ml respectively (Fig. 8). Insignificant variations were observed in case of healthy subjects at above mentioned doses of resveratrol and curcumin (Fig. 9). Thus, both, resveratrol and curcumin can be effectively used to improve the degenerating antioxidant state in the pathogenesis of osteoporosis. All values are mean $\pm$ SE, $p < 0.001$.

**5. Modulation of intramonocyte glutathione (GSH) levels.**

It is well known that intracellular signaling via NFκB is known to be ROS sensitive, and that because of high oxidative stress in osteoporosis, monocytes from the study groups (n=20 each for healthy and osteoporosis patients) were co-cultured for 24 hours with 10 mM NAC or 100 µg/ml SN50 or 100 µg/ml SN50/M or 500 ng/ml allicin or 25 µg/ml resveratrol or 25 µg/ml curcumin for comparative modulation of intramonocyte GSH levels. Control cultures (-) did not receive any treatment and revealed intramonocyte GSH levels of 321.50 as well as 134.61 pg/ml for
osteoporosis and healthy individuals respectively (Figs. 10 and 11 respectively). As evident from the results, at 24 hours, NAC, a known antioxidant, as well as SN50, an inhibitor of NFκB activation, both up-regulated the intramonocyte GSH levels in cultures of osteoporosis patients (233.67 and 258.37 pg/ml respectively). However, SN50/M, an inactive analogue of SN50, at same concentration failed to cause any modulation in osteoporosis patients (137.02 pg/ml). Next, allicin from garlic as well as resveratrol and curcumin, which are well established natural antioxidants, were chosen for this modulation study. Interestingly, all the three natural antioxidants, namely allicin, resveratrol and curcumin appreciably up-regulated the intramonocyte GSH levels in osteoporosis patients (283.33, 278.11 and 259.12 pg/ml respectively). When compared, they were much more potently than either NAC or SN50. Insignificant variation was recorded in case of healthy individuals with any of the modulating agent used here (Fig. 11). These results indicate that the down-regulated intramonocyte GSH levels in osteoporosis patients is NFκB mediated and that all the three natural antioxidants, namely allicin, resveratrol and curcumin proved as potential natural and safer antioxidants in osteoporosis. All values are mean ± SE, p < 0.001.

6. Levels of malondialdehyde (MDA) in serum of osteoporosis patients.

Next, due to the results showing impaired antioxidant systems in osteoporosis patients as revealed by decreased GPx activity and reduced GSH levels as mentioned above, the levels of a by-product of lipid peroxidation i.e., malondialdehyde (MDA) was measured in the present study, in the sera of osteoporosis patients (n=20), in order to detect further signs of increased oxidative stress. In comparison to healthy group (8.01 ng/ml), the serum MDA values were found to be almost three times higher in osteoporosis patients (25.11 ng/ml) as depicted in Fig. 12. All values are mean and p < 0.001 in each case.

7. Estimation of MDA levels in culture supernatants of monocytes.

Thereafter, adherent monocytes from PBMCs were cultured for 24 hours and supernatants analysed for MDA levels. In osteoporosis patients (n=20), MDA levels stood to a near 3.5 times (26.84 ng/ml) the level found in healthy group (n=20) (7.68 ng/ml) as depicted in Fig. 13. Data represent mean SE, p < 0.001 in each case. Hence,
osteoporotic patients are indeed exposed to high oxidative stress as indicated by high levels of MDA.

8. Effect of varying doses of Allicin on MDA levels in supernatants of cultured monocytes.

In order to find out if allicin from garlic could prove beneficial in combating/neutralizing the augmented oxidative stress which is so common in osteoporosis, monocytes obtained from the study groups were co-cultured for 24 hours with varying doses of allicin (0-500 ng/ml) and supernatants were subjected to evaluation for MDA levels. The MDA levels, ranging only from 5.15 ng/ml to 5.77 ng/ml in the supernatants of monocyte cultures of healthy subjects, remained more or less unaltered at all the concentrations of allicin used (data not shown). While in case of osteoporosis patients, the MDA levels showed a dose-dependent decrease from 24.24 ng/ml when monocytes were cultured alone through 17.23, 15.65, 10.23 to as low as 8.56 ng/ml when treated with 50, 100, 250, and 500 ng/ml allicin respectively (Fig. 14). Thus, at doses of 250 and 500 ng/ml allicin, MDA levels in osteoporosis patients were comparable to those found in healthy subjects thereby successfully upholding the potential antioxidant property of allicin in combating high oxidative stress in osteoporosis. All values are mean SE, n=20 in each study group and \( p < 0.001 \) in each case.

9. Dose response effect of Resveratrol and Curcumin on MDA levels in monocyte culture supernatants.

Next, monocytes were similarly treated for 24 hours with varying doses of resveratrol and curcumin (0-25 µg/ml) in order to explore if any positive impact resveratrol and curcumin would show in the pathogenesis of bone loss due to osteoporosis. Therefore, the supernatants of the said cultures when analyzed for MDA levels, which showed a progressive down-regulation in osteoporosis patients (n=20) from 25.32 ng/ml when monocytes were cultured alone and then through 22.67, 17.21, 12.33, 10.28, 6.12 to as only as 6.01 ng/ml when treated with 2, 5, 10, 15, 20 and 25 µg/ml of resveratrol respectively as depicted in Fig. 15. No significant variation was observed at any of the above mentioned resveratrol doses in case of healthy group with MDA levels of 8.19, 8.01, 7.67, 8.23, 7.93, 8.05 and 7.87 ng/ml respectively (Fig. 16). All values are mean
SE of n=20 in each study group and $p < 0.001$ in each case. Therefore, the said data further substantiated that resveratrol was an efficient antioxidant in osteoporosis.

Similarly, varying doses of curcumin (0-25 µg/ml) also exhibited an appreciable down-regulation in MDA levels in 24 hours culture supernatant of osteoporosis patients (Fig. 17). However, the effect was a bit lower when compared with that of resveratrol. No effect was observed in healthy controls (Fig. 18).

[B]: Immunological Study by ELISA:

1. **Estimation of IL-1β levels in sera and supernatants of monocyte cultures of healthy individuals and osteoporosis patients by ELISA.**

Since, it is an established fact that the high levels of pro-inflammatory cytokine IL-1β are found in osteoporosis patients, thus, an attempt was made to probe the levels of IL-1β in sera as well as monocyte culture supernatants of osteoporosis patients and consequently, the results were compared with those obtained from healthy subjects. ELISA results depicted in Fig. 19 show high basal levels of IL-1β in sera as well as monocyte culture supernatants of osteoporosis patients (172.26 pg/ml and 132.12 pg/ml respectively) compared to IL-1β secretion in healthy sera and monocyte cultures (25.13 pg/ml and 8.15 pg/ml respectively). All values are mean ± SE; $p < 0.001$ and n=8 in each study group.

2. **Effect of varying doses of Allicin on expression of IL-1β in monocyte culture supernatants.**

Monocytes from osteoporosis patients were co-cultured for 24 hours with varying concentrations of allicin (0, 50, 100, 250 and 500 ng/ml). Cultures were harvested and supernatants obtained were subjected to ELISA for comparative evaluation of IL-1β secretion. As is evident from Fig. 20, the secretion of IL-1β was found to decrease dose-dependently in osteoporosis patients from 151.34 pg/ml through 140.11, 40.42, 28.81 and 24.29 pg/ml at 0, 50, 100, 250 and 500 ng/ml allicin respectively. IC$_{50}$ was computed out to be in between 50-100ng/ml. In case of healthy subjects, low IL-1β secretion remained more or less unaffected, which were to the order of 4.51 pg/ml,
4.38, 5.03, 4.06 and 5.25 pg/ml respectively at the above varying doses of allicin (Fig. 21). All values are mean ± SE, \( p < 0.001 \) and \( n=8 \) in each of the study groups.

3. **Dose-response effect of Resveratrol and Curcumin on the expression of IL-1β in monocyte culture supernatants.**

Next, monocytes of study groups were similarly treated with varying concentrations of resveratrol (0, 2, 5, 10, 15, 20 and 25 \( \mu \)g/ml). A similar response was observed with resveratrol as was observed above with allicin. In case of osteoporosis patients, IL-1β secretion dose-dependently decreased from 162.34 pg/ml through 140.36, 78.59, 40.26, 27.21 and 21.09 pg/ml (Fig. 22). \( \text{IC}_{50} \) was computed out to be ~ 8 \( \mu \)g/ml. In healthy individuals, 3.89, 4.35, 3.96, 4.91, 4.88 and 5.02 pg/ml IL-1β secretion levels were recorded at the respective varying doses of resveratrol (Fig. 23). All data represent mean ± SE; \( p < 0.001 \) and \( n=8 \) in each study groups.

Similarly, curcumin showed a dose-dependent down-regulation in IL-1β levels from 165.09, 143.22, 89.32, 55.23, 35.19 and 29.12 pg/ml with 0, 2, 5, 10, 15, 20 and 25 \( \mu \)g/ml of curcumin respectively (Fig. 24). \( \text{IC}_{50} \) was computed out to be ~ 9 \( \mu \)g/ml. On the contrary, no effect of curcumin was observed on IL-1β in healthy control and monocyte culture supernatants (Fig. 25).

[C] **Osteoclast and bone marker study by TRAP, ELISA and Quantitative ‘real-time’ RT-PCR.**

1. **Determination of Human sRANKL levels in culture supernatants of healthy controls and osteoporosis patients**

An attempt was made to probe the levels sRANKL in culture supernatants of healthy controls (\( n=8 \)) and osteoporosis patients (\( n=8 \)) by ELISA. In comparison to healthy controls (\( p < 0.01 \)), patient’s cultures (5 days) exhibited around 9-fold augmented levels of sRANKL (pg/ml; \( p < 0.001 \)) (Fig. 26).

2. **Dose response effect of Allicin on sRANKL levels in culture supernatants of healthy controls and osteoporosis patients**
Adherent monocytes were co-cultured with varying concentrations of allicin from garlic (0, 50, 100, 250 and 500 ng/ml). In case of osteoporosis patients, sRANKL secretion dose-dependently decreased from 33.16 pg/ml at through 27.54, 20.33, 12.98 and 3.02 pg/ml with 50, 100, 250 and 500 ng allicin respectively (Fig. 27; \( p < 0.001 \)). On the contrary, healthy controls exhibited in between 1.33 – 3.12 pg/ml of RANKL (Fig. 28). Next, after dose response evaluation, an attempt was also made to re-check the data by co-culturing with the maximum dose of allicin (500 ng/ml) selected in the study, and that, similar results were observed to the one’s observed above in dose response experiments at the maximum dose (Fig. 28; \( p < 0.001 \)). All data represent mean ± SE; \( p < 0.001 \) and n=8 in each study groups.

3. Evaluation of Allicin-induced percent suppression by computational analysis in the secretion of sRANKL in culture supernatants of osteoporosis patients

Computation of the data depicted in Fig. 27 exhibited that allicin suppressed the secretion of sRANKL by around 16.94%, 38.69%, 60.85% and 90.89% with 50, 100, 250 and 500 ng allicin respectively (Fig. 29). The IC\(_{50}\) was computed out to be in between 100 -135 ng/ml.

4. Dose response effect of Resveratrol and Curcumin on sRANKL levels in culture supernatants of healthy controls and osteoporosis patients

Monocytes of study groups were co-cultured separately with varying concentrations of resveratrol and curcumin (0, 2, 5, 10, 15, 20 and 25 \( \mu \)g/ml). In case of osteoporosis patients, sRANKL secretion was found to dose-dependently decrease from 35.16 pg/ml at through 29.11, 24.26, 15.16, 11.33, 9.16 and 8.89 pg/ml with 0, 2, 5, 10, 15, 20 and 25 \( \mu \)g resveratrol respectively (Fig. 30; \( p < 0.001 \)). On the contrary, healthy controls exhibited in between 1.3 – 3.2 pg/ml of RANKL (Fig. 30).

Thereafter, the effect of varying doses of curcumin on RANKL in 5 days monocyte cultures was also analyzed. Co-culturing of patients’ monocytes with curcumin exhibited a dose-dependent down-regulation of sRANKL, which was of the order of 32.65, 27.81, 22.45, 17.29, 13.07, 9.44 and 9.23 pg/ml with 0, 2, 5, 10, 15, 20 and 25 \( \mu \)g/ml of curcumin respectively (Fig. 31). All data represent mean ± SE; \( p < 0.001 \) and n=8 in each study groups. IC\(_{50}\) was computed out to be ~ 9 \( \mu \)g/ml.
Next, computational analysis of the data revealed that curcumin down regulated the secretion of sRANKL by around 15%, 31%, 46%, 60%, 70% and 73% with 2, 5, 10, 15, 20 and 25 µg curcumin respectively (Fig. 32).

5. Generation of Human Osteoclast Precursors from Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were used directly for the generation of osteoclast precursors after centrifugation with Ficoll-Hypaque. After the 3 day culture duration in osteoclastogenic medium (α-MEM culture medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 ng/ml M-CSF and 25 ng/ml RANKL), multinucleated osteoclast precursors were observed to appear and the number increased after 5 days of culture, as revealed by Tartrate Resistant Acid Phosphatase (TRAP) staining (Figs. 33, 34). However, there was negligible appearance of osteoclast precursors after 24 h (1 day) of culture (Fig. 33). The number of multinucleated preosteoclasts, arising from PBMCs isolated from the blood of normal healthy individual (data not shown) and osteoporosis patients (Figs. 33, 34), were counted by TRAP staining. Interestingly, we observed an individual variation in osteoclast generation from different donors as depicted by different number of multinucleated cells in Figs. 33 and 34.

6. Effect of Allicin, Resveratrol and Curcumin on the Generation of Human Osteoclasts

Interestingly, we observed that co-culturing of PBMCs from osteoporosis patients (n=30) with Resveratrol (25 µg/ml) or Curcumin (25 µg/ml) or Allicin (500 ng/ml) in osteoclastogenic medium for 3 days resulted in an appreciable amount of reduction in appearance of multinucleated osteoclast precursors (Figs. 35, 36, 37, 38, 39, 40, 41, 42 and 43). Hence, this reflects the potential of allicin, resveratrol and curcumin to exert regulatory effect in osteoclast generation and differentiation. The above doses of allicin, resveratrol and curcumin were selected after performing dose response experiment, where TRAP assay revealed a linear suppression in the formation of multinucleated cells was observed. The above data shows nearly 20-30 % suppression in appearance of multinucleated cells in cultures separately resveratrol (25 µg/ml) or curcumin (25 µg/ml) respectively receiving relative to control devoid of any
supplement. Interestingly, around 35-40 % suppression in appearance of multinucleated cells was observed in cultures receiving 500 ng/ml of allicin relative to control cultures devoid of any allicin (Figs. 35-43).

**Effect of Natural Antioxidant, Epigallocatechin gallate (EGCG), on the Generation of Human Osteoclasts**

Interestingly, we observed that co-culturing of PBMCs with EGCG (20 µg/ml) in osteoclastogenic medium for culture duration of 5 days leads to appreciable amount of reduction in appearance of multinucleated osteoclast precursors (Fig.43 a, 43 b). Hence, this data gives an idea of potential of EGCG to exert regulatory effect in osteoclast generation and differentiation.

7. **Time course kinetics of multinucleated cell suppression by natural antioxidants**

Monocytes from osteoporosis patients (n=6) were separately co-cultured with allicin (500 ng/ml), resveratrol (25 µg/ml) and curcumin (25 µg/ml) for varying time periods i.e., for 0 hour, 24 hours, 72 hours and 120 hours. Control cultures devoid of any supplements exhibited multinucleated cells to the order of 4, 5, 73 and 101 at 0 hour, 24 hours, 72 hours and 120 hours of culture period respectively (Fig. 44). On the contrary, cultures receiving allicin (500 ng/ml), resveratrol (25 µg/ml) or curcumin (25 µg/ml) exhibited a linear increase in the suppression of multinucleated cells with increase in time period of culture (Fig. 44). Thus, in comparison to control as mentioned above, allicin showed suppression to the order of 0, 2, 16 and 20 multinucleated cells at 0 hour, 24 hours, 72 hours and 120 hours of culture (Figure 44). Similarly, resveratrol exhibited suppression to the order of 1, 3, 22 and 24, while curcumin exhibited suppression to the order of 1, 3, 27 and 27 multinucleated cells at 0 hour, 24 hours, 72 hours and 120 hours of cultures respectively (Fig. 44).

8 (a) **Determination of OPN by ELISA in sera of healthy controls and patients with osteoporosis.**

The presence of human OPN was probed both in sera of healthy controls (n=10) and osteoporosis patients (n=10). As evident from Fig. 45, sera of healthy controls showed negligible levels of osteopontin (range: 2.07 ng/ml to 3.24 ng/ml). On the contrary,
sera of osteoporosis patients exhibited appreciable levels of OPN, which was in the range of 15.98 ng/ml to 28.35 ng/ml (Fig. 46; \( p < 0.001 \)). Thus, in comparison to healthy controls, around an 8-fold OPN levels were recorded in osteoporosis patients.

(b) Determination of OPN by ELISA in monocyte culture supernatants of healthy controls and osteoporosis patients.

No or negligible levels of OPN were observed in 24 hours culture supernatants of monocytes obtained from healthy controls (range: 1.09 ng/ml to 2.36 ng/ml; n=6) (Figs. 47, 48). However, an appreciable levels/amount of OPN was detected in 24 hours culture supernatants of monocytes obtained from osteoporosis patients (range: 16.23 ng/ml to 21.29 ng/ml; n=8; \( p < 0.001 \)). Thus, in 24 hours monocyte culture supernatants, around 12 fold levels of OPN was detected in osteoporosis patients in comparison to healthy controls.

(c) Modulation Study of OPN by employing positive modulator Calcitonin (CT), in 3 days monocyte cultures of patients with osteoporosis.

Monocytes from osteoporosis patients (n=6) were cultured for 3 days in osteoclastogenic medium with and without 1 ng/ml of CT. Cultures devoid of any CT exhibited OPN levels in the range of 15.67 ng/ml to 22.17 ng/ml (Fig. 49; \( p < 0.001 \)). However, in comparison to the above cultures devoid of CT, those culture wells receiving 1ng/ml of CT exhibited an augmentation/increase by around 1.4-folds in OPN levels (range: 21.11 ng/ml to 33.45 ng/ml) (Fig. 49; \( p < 0.001 \)). This substantiates that CT was a positive modulator of OPN.

(d) Effect of Resveratrol alone as well as combination of Resveratrol and CT on OPN levels in 3 days monocyte cultures in osteoclastogenic medium.

Here again, monocytes from osteoporosis patients (n=6) were cultured for 3 days in osteoclastogenic medium along with either resveratrol (20 \( \mu \)g/ml) alone or with a combination of resveratrol (20 \( \mu \)g/ml) + CT (1 ng/ml).

As evident from Fig. 50, an appreciably down-regulated/suppressed levels of OPN was recorded in monocyte cultures receiving resveratrol (20 \( \mu \)g/ml), which was in the
range of 4.13 ng/ml to 6.99 ng/ml ($p < 0.001$). Furthermore, cultures receiving a combination of resveratrol and CT as said above, suppressed OPN levels which were in the range of 9.43 ng/ml to 10.55 ng/ml (Fig. 50; $p < 0.001$). Comparison of this data with the one’s obtained in Fig. 49, where CT was found to up-regulate OPN, here in Fig. 50, the results clearly shows that the positive modulatory effect of CT on OPN was appreciably neutralized by resveratrol, thereby exerting a remarkable negative modulatory effect on OPN.

**(e) Effect of Curcumin alone as well as a mixture of Curcumin and CT on OPN levels in 3 days monocyte cultures in osteoclastogenic medium.**

Monocytes from osteoporosis patients (n=6) were cultured for 3 days as has been described with resveratrol previously. Here, cultures separately received curcumin (20 µg/ml) or a mixture of curcumin (20 µg/ml) and CT (1 ng/ml).

As evident from Fig. 51, cultures receiving curcumin alone exhibited an appreciable suppression in OPN levels (range: 8.88 ng/ml to 10.41 ng/ml; $p < 0.001$) but this suppression was slightly lesser than those observed with resveratrol in Fig. 50. Next, cultures receiving a combination of curcumin (20 µg/ml) + CT (1 ng/ml) exhibited similar results (Fig. 51) as was observed previously with combination of resveratrol + CT in Fig. 50. Thus, the results here in Fig. 51 clearly show that curcumin was a potent negative modulator of OPN but to a slightly lesser degree than resveratrol.

**(f) Effect of Allicin from garlic as well as a mixture of Allicin and CT on OPN levels in 3 days monocyte cultures in osteoclastogenic medium.**

Next, we probed the effect of the third natural antioxidant, namely allicin, in the present study on OPN levels. Therefore, monocytes from osteoporosis patients (n=6) were cultured exactly similar to the manner as discussed above for resveratrol and curcumin. In this study, cultures separately received allicin (500 ng/ml) and a combination of allicin (500 ng/ml) and CT (1 ng/ml). Thus, as evident from Fig. 52, allicin (500 ng/ml) was found to suppress OPN to a appreciably high magnitude, wherein, the range of suppression of OPN in various patients was in between 3.89 ng/ml and 5.98 ng/ml (Fig. 52; $p < 0.001$) in comparison to cultures devoid of any allicin (Fig. 49). Furthermore, culturing of monocytes with a combination of allicin
(500 ng/ml) and CT (1 ng/ml) again showed remarkable suppression of OPN and that the positive modulator CT had no substantial effect in presence of the negative modulator i.e., allicin (Fig. 52). Next, upon comparison of the data depicted in Figs. 49-52, it was observed that allicin proved to be the most potent suppressor of OPN followed by resveratrol, and in turn, followed by curcumin.

(g) Effect of Denosumab (Prolia) on OPN levels in 3 days monocyte cultures in osteoclastogenic medium.

Next, the effect of known negative modulator, namely Denosumab (Prolia) of OPN and osteoclasts was probed. As evident from Fig. 53, monocyte cultures of osteoporosis patients (n=6) receiving denosumab (Prolia) (1 ng/ml) for 3 days in osteoclastogenic medium exhibited a remarkably suppressed/ down-regulated levels of OPN (range: 3.99 ng/ml to 5.23 ng/ml; p < 0.001) in comparison to cultures devoid of any supplements (Fig. 49). Upon comparative analysis of the data obtained in Figs. 49-53, it was observed that the degree of suppression/ down-regulation of OPN was nearly similar/ same with natural antioxidant allicin and monoclonal antibody namely denosumab (Prolia).

10. A study on Bone Marker by Quantitative ‘Real time RT-PCR:

Next, an attempt was made for characterization of monocytes at the gene level from osteoporosis patients and compared to those of healthy individuals with respect to bone markers like TNF-α.

(a) Expression of TNF-α mRNA

As described in methods, PBMCs were isolated from the blood of normal healthy individuals and osteoporosis patients, and in turn, were subjected to adherent monocytes isolation. Monocytes from osteoporosis patients were then subjected to TNF-α mRNA evaluation by real-time RT-PCR. Thereafter, they were compared with the values of TNF-α mRNA copy number recorded in monocytes from healthy subjects. As is evident from Fig. 54, in comparison to healthy subjects’ monocytes, those from osteoporosis patients revealed the presence of high basal levels of TNF-α mRNA copy number which was to the order of 8.33E+08 (p < 0.001). These data,
therefore, revealed significantly high basal levels of TNF-\(\alpha\) mRNA in the monocytes of osteoporosis patients. All values are mean ± SE of six experiments in each study group.

(b) Expression of OPG mRNA.

Furthermore, mononuclear cells from the study group were subjected to OPG mRNA evaluation by real-time RT-PCR. In case of osteoporosis patients, the data exhibited higher basal levels of OPG mRNA copy number which was \(\sim 8.9\) logs \((p < 0.001)\) than the healthy subjects level (Fig. 55). This indicated higher basal level expression of OPG mRNA in osteoporosis patients’ PBMCs.

(c) Dose response effect of Allicin from garlic, Resveratrol and Curcumin on human housekeeping gene R18.

Because of well established health benefits of garlic, resveratrol and curcumin since ancient times, we chose here to study their actions on monocytes isolated from PBMCs of osteoporosis patients (n=6). Allicin or resveratrol and curcumin failed to show any adverse effect on the human housekeeping gene R18. As evident from Fig. 56-58 that neither allicin (0-500 ng/ml) nor resveratrol (0-20 \(\mu\)g/ml), nor curcumin (0-20 \(\mu\)g/ml) at any of their respective concentrations when these were used to co-culture the monocytes, had any significant effect on the expression of human housekeeping gene R18 as revealed by quantitative real-time RT-PCR (Fig. 56-58).

(d) Effect of varying doses of Allicin, Resveratrol and Curcumin on TNF-\(\alpha\) expression.

We first started with allicin to investigate its action upon TNF-\(\alpha\) mRNA gene expression. Adherent monocytes from PBMCs of osteoporosis patients and healthy subjects were co-cultured with varying concentrations of allicin (0-500 ng/ml) for 24 hours. Cultures devoid of allicin i.e., at 0 ng served as control. Monocytes were then subjected to TNF-\(\alpha\) mRNA evaluation by real-time RT-PCR. It is evident from Fig. 59, the expression of TNF-\(\alpha\) mRNA was dose-dependent showing significant down regulation in its copy number in case of osteoporosis patients from 8.33E+08 in untreated monocytes through 0.64 logs, \(\sim 4.1\) logs, \(\sim 5.0\) logs to \(\sim 6.1\) logs when
monocytes were treated with 50, 100, 250, and 500 ng/ml allicin respectively. However, no significant variation was observed in case of healthy individuals (n=6) (Fig. 59). The data, thus prove allicin as a potent suppressor of augmented TNF-α mRNA levels in osteoporosis whereby it can be used as a potent anti bone-resorptive agent in this kind of bone pathogenesis. Data represent mean ± SE of six experiments in each study group i.e., n=6 and p < 0.001 in each case.

Thereafter, it was also attempted to probe the effect of varying concentrations of resveratrol (0-20 µg/ml) and curcumin (0-20 µg/ml) on the expression of TNF-α mRNA in 24 hours cultures of PBMCs isolated from osteoporosis patients and healthy subjects. As is evident in Fig. 60, monocytes when subjected to TNF-α mRNA evaluation by real-time RT-PCR showed dose-dependence on resveratrol and curcumin respectively in all study groups. In case of osteoporosis patients, significant down regulation of TNF-α mRNA copy number of as much as 0.35 logs, 3.6 logs, ~ 5.1 logs, 5.5 logs and 5.8 logs was observed in monocytes treated with 2, 5, 10, 15 and 20 µg/ml of resveratrol respectively when compared to untreated monocytes.

On the contrary, no significant change in gene expression was observed in case of healthy subjects n=6) at any of the concentrations of resveratrol used. Similar observations were observed with curcumin in both healthy and patients’ cultures (Fig. 60). The data, thus points to resveratrol and curcumin especially at 15 and 20 µg/ml doses, as effective natural immunoregulator that can be used against the pathogenesis of bone loss in osteoporosis. All values are mean ± SE of six experiments (n=6) (p < 0.001).
Figure 1

Fig. 1: Oxidative Stress Study in Serum by Determining the GPx Activity in healthy controls (n=30) and osteoporosis patients (n=30). The data represents mean ± S.E.M.; p<0.001.
Fig. 2: Oxidative Stress Study in Monocyte Cultures by Determining the GPx Activity in Healthy Controls (n=200) and Osteoporosis Patients (n=20). The data represents mean ± S.E.M.; $p<0.001$. 
Fig. 3: Determination of Intramonomocyte Glutathione [GSH] (pg/ml) levels in 24 hr monocyte cultures of healthy controls (n=20) and osteoporosis patients (n=20). The data represents mean ± S.E.M.; p<0.001.
Figure 4

Fig. 4: Dose response effect of Curcumin (0 to 25 µg/ml) on GPx activity in 24 hr monocyte cultures of healthy controls (n=20). The data represents mean ± S.E.M.; $p<0.001$. (Black bars = Resveratrol; Blue bars = curcumin).
Figure 5

Fig. 5: Dose response effect of Curcumin (0-25 µg/ml) on GPx activity in 24 hr monocyte culture supernatants of osteoporosis patients' (n=20). The data represents mean ± S.E.M.; p<0.001.
Fig. 6: Dose response effect of Resveratrol (0-25 µg/ml) on GPx activity in 24 hr monocyte culture supernatants of osteoporosis patients (n=20). The data represents mean ± S.E.M.; $p<0.001$. 
Fig. 7: Dose response effect of allicin (0-500 ng/ml) on intramonomocyte GSH in 24 hr monocyte cultures of healthy (n=20) and osteoporosis patients (n=20). The data represents mean ± S.E.M.; $p<0.001$. (Black bars = healthy; Blue bars = patients).
Fig. 8: Dose response effect of Resveratrol (0-25 µg/ml) and Curcumin (0-25 µg/ml) on intramonlyte GSH levels in 24 hr monocyte cultures of osteoporosis patients. The data represents mean ± S.E.M.; \( p<0.001 \). (Black bars = controls which did not receive any supplements; Red bars = patients culture receiving resveratrol; and blue bars = patients culture receiving curcumin).
Fig. 9: Dose response effect of Resveratrol (0-25 µg/ml) and Curcumin (0-25 µg/ml) on intramonocyte GSH levels in 24 hr monocyte cultures of healthy controls. The data represents mean ± S.E.M.; *p<0.001. (Black bars = resveratrol; Blue bars = Curcumin).
Fig. 10: Modulation of intramonomocyte GSH levels in 24 hr monocyte cultures of Osteoporotic Patients (n=20). The supplements used for modulation were NAC (10 mM), SN50 (100 µg/ml), SN50/M (100 µg/ml), Allicin (500 ng/ml), Resveratrol (25 µg/ml), Curcumin (25 µg/ml) and (-) represents cultures devoid of ant treatment. The data represents mean ± S.E.M.; p<0.001.
Fig. 11: Modulation of intramonomocyte GSH levels in 24 hr monocyte cultures of Healthy Control (n=20). The supplements used for modulation were NAC (10 mM), SN50 (100 µg/ml), SN50/M (100 µg/ml), Allicin (500 ng/ml), Resveratrol (25 µg/ml), Curcumin (25 g/ml) and (-) represents cultures devoid of ant treatment. The data represents mean ± S.E.M.; p<0.001.
Fig. 12: Determination of serum Malondialdehyde (MDA) Levels, ng/ml (n=20 for each study group). The data represents mean ± S.E.M.; p<0.001.
Fig. 13: Determination of MDA levels in 24 hr culture supernatants of monocytes of healthy controls (n=20) and osteoporosis patients (n=20). The data represents mean ± S.E.M.; $p<0.001$. 
Fig. 14: Dose response effect of Allicin on MDA levels in 24 hr monocyte culture supernatants of Osteoporosis Patients (n=20). The data represents mean ± S.E.M.; $p<0.001$. 
Figure 15

Fig. 15: Dose response effect of Resveratrol on MDA levels in 24 hr monocyte culture supernatants of osteoporotic patients (n=20). The data represents mean ± S.E.M.; p<0.001.
Fig. 16: Dose response effect of Resveratrol on MDA levels in 24 hr monocyte culture supernatants of Healthy Controls (n=20). The data represents mean ± S.E.M.; \( p<0.001 \).
Fig. 17: Dose response effect of Curcumin on MDA levels in 24 hr monocyte culture supernatants of Osteoporosis Patients (n=20). The data represents mean ± S.E.M.; $p<0.001$. 
Fig. 18: Dose response effect of Curcumin on MDA levels in 24 hr monocyte culture supernatants of Healthy Control (n=20). The data represents mean ± S.E.M.; p<0.001.
Fig. 19: Levels of IL-1 Beta in serum and 24 hr monocyte culture supernatants of healthy controls (n=8) and osteoporosis patients (n=8). The data represents mean ± S.E.M.; $p<0.001$. 
Fig. 20: Dose-response effect of allicin (0-500 ng/ml) on expression of IL-1β in 24 hr monocyte culture supernatants of Osteoporosis Patients (n=8). The data represents mean ± S.E.M.; $p<0.001$. 
Fig. 21: Dose-response effect of allicin (0-500 ng/ml) on expression of IL-1β in 24 hr monocyte culture supernatants of Healthy Controls (n=8). The data represents mean ± S.E.M.; p<0.001.
Fig. 22: Dose-response effect of Resveratrol (0-25 µg/ml) on expression of IL-1β in 24 hr monocyte culture supernatants of Osteoporosis Patients (n=8). The data represents mean ± S.E.M.; p<0.001.
Fig. 23: Dose-response effect of Resveratrol (0-25 µg/ml) on expression of IL-1β in 24 hr monocyte culture supernatants of Healthy Controls (n=8). The data represents mean ± S.E.M.; $p<0.001$. 

**Figure 23**
Fig. 24: Dose-response effect of Curcumin (0-25 µg/ml) on expression of IL-1β in 24 hr monocyte culture supernatants of Osteoporosis Patients (n=8). The data represents mean ± S.E.M.; p<0.001.
Fig. 25: Dose-response effect of Curcumin (0-25 µg/ml) on expression of IL-1β in 24 hr monocyte culture supernatants of Healthy Controls (n=8). The data represents mean ± S.E.M.; *p<0.001.
Figure 26

Fig. 26: Determination of Human RANKL levels in 24 hr monocyte culture supernatants of healthy controls (n=8) and osteoporosis patients (n=8) by ELISA. The data represents mean ± S.E.M.; \( p<0.001 \).
Fig. 27: Dose response effect of Allicin (0-500 ng/ml) on human RANKL in five days monocyte culture supernatants of Osteoporosis patient (n=8). The data represents mean ± S.E.M.; $p<0.001$. 
Fig. 28: ELISA for determining the level of Human RANKL in five days monocyte culture supernatants of various Healthy Controls (n=8) and patient (n=8) that were co-cultured with 500 ng/ml of allicin. The data represents mean ± S.E.M.; $p<0.001$. 
Fig. 29: Determination of varying doses of Allicin-induced percent inhibition of RANKL by computational analysis. $p<0.001$. 
Fig. 30: ELISA for determining the level of Human RANKL in five days monocyte culture supernatants of various healthy (n=8) and patient (n=8) that were co-cultured with varying doses (0 - 25 µg/ml) of Resveratrol. The data represents mean ± S.E.M.; $p<0.001$. (Black bars = healthy controls; blue bars = patients).
Fig. 31: Dose response effect of Curcumin (0-25 µg/ml) on human RANKL in five days monocyte culture supernatants of Osteoporosis patient (n=8). The data represents mean ± S.E.M.; $p<0.001$. 

Figure 31
Fig. 32: Determination of Curcumin-induced percent inhibition of RANKL by computational analysis.
Fig. 33: Generation of multinucleated cells in 24 hrs (1 day) and 72 hrs (3 days) monocyte cultures of individual osteoporotic patients’ (patient numbers 1 to 10) under Osteoclastogenic medium. $p<0.001$; (Black bars = 72 hrs i.e. 3 days culture; blue bars = 24 hr i.e. 01 day culture).
Fig. 34. Generation of multinucleated cells in 120 hrs (5 days) monocyte cultures of individual osteoporotic patients’ (patient numbers 1 to 10) under Osteoclastogenic medium. $p<0.001$. 
Fig. 35: Effect of allicin (500 ng/ml) on the generation of multinucleated cells in 72 hrs (3 days) monocyte cultures of individual osteoporotic patients’ (patient numbers 1 to 10). $p<0.001$; (Black bars = without allicin; blue bars = with allicin).
Fig. 36: Effect of allicin (500 ng/ml) on the generation of multinucleated cells in 72 hrs (3 days) monocyte cultures of individual osteoporotic patients’ (patient numbers 11 to 20). $p<0.001$; (Black bars = without allicin; blue bars = with allicin).
Fig. 37: Effect of allicin (500 ng/ml) on the generation of multinucleated cells in 72 hrs (3 days) monocyte cultures of individual osteoporotic patients’ (patient numbers 21 to 30). *p<0.001*; (Black bars = without allicin; blue bars = with allicin).
Fig. 38: Effect of Resveratrol (25 µg/ml) on the generation of multinucleated cells in 72 hrs (3 days) monocyte cultures of individual osteoporotic patients’ (patient numbers 1 to 10). $p<0.001$; (Black bars = without resveratrol; blue bars = with resveratrol).
Fig. 39: Effect of Resveratrol (25 μg/ml) on the generation of multinucleated cells in 72 hrs (3 days) monocyte cultures of individual osteoporotic patients’ (patient numbers 11 to 20). p<0.001; (Black bars = without resveratrol; blue bars = with resveratrol).
Fig. 40: Effect of Resveratrol (25 µg/ml) on the generation of multinucleated cells in 72 hrs (3 days) monocyte cultures of individual osteoporotic patients’ (patient numbers 21 to 30). $p<0.001$; (Black bars = without resveratrol; blue bars = with resveratrol).
Fig. 41: Effect of Curcumin (25 µg/ml) on the generation of multinucleated cells in 72 hrs (3 days) monocyte cultures of individual osteoporotic patients’ (patient numbers 1 to 10). *p*<0.001; (Black bars = without curcumin; blue bars = with curcumin).
Fig. 42: Effect of Curcumin (25 µg/ml) on the generation of multinucleated cells in 72 hrs (3 days) monocyte cultures of individual osteoporotic patients’ (patient numbers 11 to 20). $p<0.001$; (Black bars = without curcumin; blue bars = with curcumin).
Fig. 43: Effect of Curcumin (25 g/ml) on the generation of multinucleated cells in 72 hrs (3 days) monocyte cultures of individual osteoporotic patients’ (patient numbers 21 to 30). $p<0.001$; (Black bars = without curcumin; blue bars = with curcumin).
Fig. 43a: Individual variation in osteoclast generation in 72 hr monocyte cultures from different donors. Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) as described under Methodology. The results from 10 patient donors are presented as quantification of TRAP-positive multinucleated cell number. The effect of EGCG (20 µg/ml) is shown in black bars; $p<0.001$. 
Fig. 43b: Individual variation in osteoclast generation in 120 hrs monocyte cultures from different donors. Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) as described under Methodology. The results from 10 patient donors are presented as quantification of TRAP-positive multinucleated cell number. The effect of EGCG (20 µg/ml) is shown in black bars; \( p < 0.001 \).
Fig. 44: Time course kinetic of multinucleated cells generation in 72 hr monocyte cultures of osteoporosis patients (n=6) and their suppression in the presence of allicin (500 ng/ml), resveratrol (25 µg/ml) and curcumin (25 µg/ml); \( p<0.001 \). (Black bar=without supplements; Blue bar= With allicin (500 ng/ml), Red bar=With Resveratrol (25 µg/ml) and Violet bar= With Curcumin (25 µg/ml).
Fig. 45: OPN levels (ng/ml) determined by ELISA in normal healthy sera (n=10); p<0.001.
Fig. 46: OPN levels (ng/ml) determined by ELISA in individual osteoporotic patients' sera (n=10); $p<0.001$. 
Figure 47

Fig. 47: ELISA for determining OPN levels (ng/ml) in 72 hr monocyte culture supernatant of normal healthy samples (n=6); $p<0.001$. 
Fig. 48: ELISA for determining OPN levels (ng/ml) in 72 hr monocyte culture supernatant of osteoporotic patients (n=6); $p<0.001$.  

Figure 48
Fig. 49: ELISA for determining OPN levels (ng/ml) in the absence and presence of calcitonin (1 ng/ml) in 72 hr monocyte culture supernatants of osteoporosis patients (n=6); p<0.001; [black bars = cultures devoid of calcitonin; blue bars = cultures receiving 1 ng/ml calcitonin].
Fig. 50: Determination of OPN levels (ng/ml) by ELISA in 72 hr monocyte culture supernatants of osteoporosis patients (n=6) that were co-cultured separately (a) in the presence of 25 µg/ml of Resveratrol and (b) with a mixture of 25 µg/ml Resveratrol + 1 ng/ml calcitonin. p<0.001; (Black bars = cultures receiving resveratrol only; blue bars = cultures receiving with a mixture of 25 µg/ml Resveratrol + 1 ng/ml calcitonin).
Fig. 51: Determination of OPN levels (ng/ml) by ELISA in 72 hr monocyte culture supernatants of osteoporosis patients (n=6) that were co-cultured separately (a) in the presence of 25 μg/ml of Curcumin and (b) with a mixture of 25 μg/ml Curcumin + 1 ng/ml calcitonin. p<0.001; (Black bars = cultures receiving Curcumin only; blue bars = cultures receiving with a mixture of 25 μg/ml Curcumin + 1 ng/ml calcitonin).
Fig. 52: Determination of OPN levels (ng/ml) by ELISA in 72 hr monocyte culture supernatants of osteoporosis patients (n=6) that were co-cultured separately (a) in the presence of 500 ng/ml of Allicin and (b) with a mixture of 500 ng/ml of Allicin + 1 ng/ml calcitonin. $p<0.001$; (Black bars = cultures receiving allicin only; blue bars = cultures receiving with a mixture of 500 ng/ml of Allicin + 1 ng/ml calcitonin).
Fig. 53: Determination of that were Effect of) on OPN levels (ng/ml) by ELISA in 72 hr monocyte cultures of osteoporotic patients (n=6) that were co-cultured with Denosumab (Prolia) (1 ng/ml); \( p < 0.001 \).
Fig. 54: Real Time RT-PCR for TNF-alpha mRNA copies number expression in 24 hr monocyte cultures of healthy (n=6) and osteoporosis patients (n=6). $p<0.001$. 
Fig. 55: Real Time RT-PCR for OPG mRNA expression in 24 hr monocyte cultures of healthy (n=6) and osteoporosis patients (n=6). $p<0.001$. 
Fig. 56: Dose Response effect of Allicin (0-500 ng/ml) on the Expression of Human House Keeping Gene R18 in 24 hr monocyte cultures of osteoporosis patients (n=6) by ‘real time’ RT-PCR. $p<0.001$. 
Figure 57

Fig. 57: Dose Response Effect of Resveratrol (0-20 ug/ml) on the Expression of Human House Keeping Gene R18 mRNA in 24 hr monocyte cultures of osteoporosis patients (n=6) by ‘real time’ RT-PCR., $p<0.001$. 
Fig. 58: Dose Response Effect of Curcumin (0-20 µg/ml) on the Expression of Human House Keeping Gene R18 mRNA in 24 hr monocyte cultures of osteoporosis patients (n=6) by ‘real time’ RT-PCR.. $p<0.001$. 

Figure 58
Fig. 59: Dose Response Effect of Allicin (0-500 ng/ml) on the expression of TNF-alpha mRNA in 24 hr cultures of monocytes of osteoporosis patients (n=6) by ‘real time’ RT-PCR. $p<0.001$. (Black bars = healthy; blue bars = patient).
Fig. 60: Dose Response Effect of Resveratrol (0-20 µg/ml) on the expression of TNF-alpha mRNA in 24 hr cultures of monocytes of osteoporosis patients (n=6) by ‘real time’ RT-PCR. $p<0.001$. (Black bars = healthy; blue bars = patient).
Fig. 61: Dose Response Effect of Curcumin (0-20 µg/ml) on the expression of TNF-alpha mRNA in 24 hr cultures of monocytes of osteoporosis patients (n=6) by ‘real time’ RT-PCR. $p<0.001$. (Black bars = healthy; blue bars = patient).